

Q<sup>b</sup>  
55. (NEW) A kit for measuring the transfer of energy *in vivo* or *in vitro* and containing at least one of the polynucleotides of claim 9 and the reagents necessary for visualizing or detecting the said transfer in presence or in absence of a molecule of interest.

56. (NEW) A polynucleotide encoding a fusion protein as claimed in claim 50.

### REMARKS

Entry of this Amendment prior to examination is respectfully requested.

The amendment to paragraph 16, on page 5 of the specification, changes a misspelling and an error in translation. The term "permitting" has been amended to read "transmitting," and the word "measurable" has been changed so that it is spelled correctly. These changes are also reflected in the amendment to claim 2. Support for the term "transmit" can be found on page 13, in the third sentence of paragraph 59, which recites: "Thus after the reconstitution of aequorin and its binding with calcium ions, the activated aequorin *transmits* its energy to the GFP, which in turn emits a green light to return to its ground state." (emphasis added)

The amendment to paragraph 78 corrects typographical errors in the sequences that appear on page 18 of the specification. These changes do not add new matter, but rather make the sequences conform to information already present in the paragraph. Specifically, the last codon of sequence oGM1 and pEGFP-CIdKS is GAG, which codes for Glu not Asp, as originally written. The amendment reflects this correction. Similarly, the fourth codon of sequence oGM2 is AAC, and is correctly labeled with the amino acid Asn. This amino acid label also corresponds to the fourth codon of pEGFP-CIdKS, but that codon was mistakenly written AAG, instead of AAC. The amendment only changes this typographical error. The Amendments to the amino acid labels above oGM3 merely make the amino acid and DNA

sequences match. The sequences of the first two codons were exchanged to correctly match the codon ATC with the indicated amino acid label Ile and the codon ACT with the indicated amino acid label Thr. The amino acid label Asn was changed to Gly to correctly label the codon GGC. Finally, the last codon of the pEGFP-CIdKS sequence in this set was mistakenly written ATC. It has been amended to ATG, which corresponds with the codon from oGM3. This change also makes the new codon correspond correctly to the amino acid label Met above it.

The specific amendment to claim 46 was done to correct a typographical error in the SEQ ID NO. Claim 46 recited SEQ ID NO: 251 as originally filed, but this SEQ ID NO does not exist. Instead, the sequence recited in the claim corresponds to SEQ ID NO: 25 (see Specification at pg. 6, paragraph 25), which is reflected in the amendment.

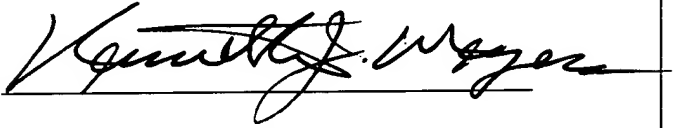
All of the other amendments to the claims were made to conform to United States patent practice, or to include SEQ ID NOS. No new matter was added to the claims. The new claims were derived from the originally filed claims.

If there is any fee due in connection with the filing of this Preliminary Amendment, please charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, L.L.P.

Dated: November 13, 2001

By: 

Kenneth J. Meyers  
Reg. No. 25,146  
Phone: (202)408-4033  
Fax: (202)408-4400  
Email: meyersk@finnegan.com

**Appendix to the Amendment of November 13, 2001**

Please note, to avoid confusion with material in the specification originally underlined, material added in this amendment is indicated in bold. Material that is to be deleted is enclosed in brackets. This system is an "equivalent marking system" to the system described in 37 C.F.R. §121(c)(1)(ii), and therefore it complies with the rule.

Please enter the following amendments.

**IN THE SPECIFICATION**

Please replace paragraph 16 on page 5 of the specification with the following paragraph:

[016] In addition, this invention provides a composition comprising a purified polypeptide, wherein the composition has the functional characteristics of binding calcium ions and [permitting measureable] **transmitting measurable** energy, said energy depending [of] **on** the quantity of calcium bound and [of] **on** the quantity of polypeptides in said composition in absence of any light excitation.

Please replace paragraph 77, on page 17 of the specification:

[077] Double digestion of pEGF-C1 plasmid (Clontech, see figure) with *KpnI* and *SmaI* enzymes. After blunt ending the *KpnI* extension with "Mung bean" nuclease, the two extremities are ligated.

5'	GTC	GAC	GCT	ACC	GCG	GGC	CCG	GGA	TCC	3'
3'	CAG	CTG	<u>CCA</u>	<u>TGG</u>	CGC	<u>CCG</u>	<u>GGC</u>	<u>CCT</u>	AGG	5'
			<i>KpnI</i>				<i>SmaI</i>			

(SEQ ID NO: 27)

↓

GTC	GAC	GGT	AC		G	GGA	TCC
-----	-----	-----	----	--	---	-----	-----

CAG	CTG	C			C	CCT	AGG
			↓				
GTC	GAC	G			G	GGA	TCC
CAG	CTG	C			C	CCT	AGG
			↓				
		GTC	GAC	GGG	GAT	CC	
		CAG	CTG	CCC	CTA	GC	
		SalI		BamHI			

(SEQ ID NO: 28)

Please replace paragraph 78 of the specification, which bridges pages 17 and 18, with the following paragraph:

Stage 2: pEGFP-C1mut (GFP mutagenesis)

[078] Four mutagenesis oligonucleotides were used on a single-stranded molecule prepared using pEGFP-C1dKS. Each oligonucleotide comprises one or several mismatches (identified below in lower case letters), causing the desired mutation. In the pEGFP-C1mut plasmid chosen, cut with the *SacII* enzyme but not the *AgeI* enzyme, all of the mutations were verified by sequencing.

-Destruction of the *AgeI* site, introduction of a *SacII* site and deletion of a Valine codon normally absent in "wild-type" GFP (Prasher, D.C., Eckenrode, R.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J., Primary structure of the *Aequorea victoria* green-flourescent protein. Gene 111 (1992) 229-233.)

			Met		Ser	Lys	Gly	[Asp]	Glu	
(SEQ ID NO: 30)		<u>SacII</u>								
oGM1:	5 - '	GCGCTACCGcggGCCACC	ATG		AGC	AAG	GGC	GAG	3'	
(SEQ ID NO.: 29)										
pEGFP-C1dKS:	5'	GCGCTACCGGTGCACC	ATG	GTG	AGC	AAG	GGC	GAG	3'	
		<u>AgeI</u>								
(SEQ ID NO: 31)				Val						

-Replacement of the 163 Valine codon by an Alanine codon in order to increase the quantity of GFP assuming a correct conformation at 37°C (Siemering, K.R., Golbik, R., Sever, R., and Haseloff, J., Mutations that suppress the thermosensitivity-of green fluorescent protein. Current Biol. 6(1996) 1653-1663.)

			Ile	Lys	Ala	Asn	Phe	Lys	
(SEQ ID NO: 34)									
oGM2:	5'	GC	ATC	AAG	Gcc	AAC	TTC	AAG	3'
(SEQ ID NO: 33)									
pEGFP-C1dKS:	5'	GC	ATC	AAG	GTG	AA[G]C	TTC	AAG	3'
(SEQ ID NO: 35)									
(SEQ ID NO: 36)					Val				

-Replacement of a 231 Leu codon by a Histidine codon normally present in "wild-type" GFP (Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J., Primary structure of the Aequorea victoria green-fluorescent protein. Gene 111 (1992) 229-233.)

			Ile	Thr	His	[Asn] Gly	Met	
(SEQ ID NO: 38)								
oGM3:	5'	GG A[CT] TC	A[TC] CT	CaC	GGC	ATG	GA	3'
(SEQ ID NO: 37)								
pEGFP-C1dKS:	5'	GG A[CT] TC	A[TC] CT	CTC	GGC	AT[C] G	GA	3'
(SEQ ID NO: 39)								
(SEQ ID NO: 40)					Leu			

Please replace paragraph 79 of the specification, on pages 18-20, with the following paragraph:

[079] Four PCRs (Polymerase Chain Reaction) done on a vector comprising the aequorin (Aeq) coding phase makes it possible to amplify the A, B, C, and D

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT,  
& DUNNER, L.L.P.  
1300 I STREET, N. W.  
WASHINGTON, DC 20005  
202-408-4000

fragments with, respectively, the primers oAE5A and oAE3A, oAE5B and oAE3B, oAE5C and oAE3C, oAE5D and oAE3D. The overlapping regions are used to assemble the different parts during successive PCRs (Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. Site-directed mutagenesis by overlap extension using the polymerase chain reaction *Gene* 77 (1989) 51-49.) An A+B fragment is amplified starting with a mixture of A and B fragments, and primers oAE5A and oAE3B. Similarly, a C+D fragment is amplified with a mixture of C and D fragments, using the primers oAE5A and oAE3D. Finally, the complete coding phase, A+B+C+D is developed with the primers oAE5A and oAE3D.

- Each oligonucleotide comprises one or several mismatches that are identified below in lower case. The “wild” sequence is represented opposite, in upper case. The primer oAE5A suppresses the original initiation translation code (ATG) and introduces a *Bgl*II site. The primer oAE3D introduces an *Xho*I site just behind the translation terminal codon (TAA). The final PCR product, digested with the *Bgl*II and *Xho*I enzymes, is cloned in the *Bgl*II-*Sa*II sites of the pEGFP-C1mut plasmid in such a way that the Valine codon (GTC), the first codon of aequorin, is the same reading phase as the GFP (see figure). The other primers introduce “silent” mutations that do not change the protein sequence but modify six codons in the jellyfish, *Aequoria Victoria*, to improve their expression in mammals (Wada, K-N., Aota, S.-I., Tshuchiya, R., Ishibashi, F., Gojobori, T., and Ikemura, T. Codon usage tabulated from the GenBank genetic sequence data.

Nucleic Acids Res. 18 suppl. (1990) 2367-2411). The completeness of  
the entire sequence was verified by sequencing,

oAE5A CCATG

5' AGCTTCagatct GTC AAA CTT ACA TCA GAC TTC GAC AAC CCA AGA TGG ATT GGA CGA

3' TCGAAGTctaca CAG TTT GAA TGT AGT CTG AAG CTG TTG GGT TCT ACC TAA CCT GCT

BG111

CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC AAC CAC AAT GGA AAA ATC TCT CTT GAC GAG

GTG TTC GTA TAC AAG TTA AAG GAA CTA CAG TTG GTG TTA CCT TTT TAG AGA GAA CTG CTC

ATG GTC TAC AAG GCA TCT GAT ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC

TAC CAG ATG TTC CGT AGA CTA TAA CAG TAG TTA TTG GAA CCT CGT TGT GGA CTC GTT CGG

oAE5B A

AAA CGA CAC AAA GAT GCT GTg GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT GGT GTG

TTT GCT GTG TTT CTA CGA CAc CTT CGG AAG AAG CCT CCT CGA CCT TAC TTT ATA CCA CAC

T oAE3A

GAA ACT GAT TGG CCT GCA TAT ATT GAA GGA TGG AAA AAA TTG GCT ACT GAT GAA TTG GAG

CTT TGA CTA ACC GGA CGT ATA TAA CTT CCT ACC TTT TTT AAC CGA TGA CTA CTT AAC CTC

oAE5C G T A

AAA TAC GCC AAA AAC GAA CCA ACC CTC ATC CGc ATc TGG GGT GAT GCT TTG TTT GAT ATC

TTT ATG CGG TTT TTG CTT GGT TGg GAG TAG GCg TA<sub>g</sub> ACC CCA CTA CGA AAC AAA CTA TAG

C A T oAE3B

GTT GAC AAA GAT CAA AAT GGA GCT ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT

CAA CTG TTT CTA GTT TTA CCT CGA TAA TGT GAC CTA CTT ACC TTT CGT ATG TGG TTT CGA

GCT GGT ATC ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT ATT GAT

CGA CCA TAG TAG GTT AGT AGT CTT CTA ACG CTC CTT TGT AAG TCT CAC ACG CTA TAA CTA

oAE5D A T A

GAA AGT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CA<sub>g</sub> CAT cT<sub>g</sub> GGA TTT TGG TAC ACC

CTT TCA CCT GTT GAG CTA CAA CTA CTC TAC TGT TCT GTc GTA gAc CCT AAA ACC ATG TGG

T A T oAE3C



XhoI

ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA GCT GTC CCC TAA TCTcGAGGATCTTT 3'

TAC CTA GGA CGA ACG CTT TTC GAG ATG CCA CCT CGA CAG GGG ATT AGAgCTCCTAGAAA 5'

T oAE3D

(SEQ ID NO: 41)

Please replace paragraph 80, bridging pages 20 and 21 of the specification, with the following paragraph:

[080] In the pEGFPmur-Aeq plasmid, a sequence of five amino acids exists between the coding phases of the GFP and aequorin. Observations led to the lengthening of this region by intercalating a sequence in the *BspEI* site. Two complementary oligonucleotides coding for a sequence of nine amino acids give the composition a good deal of flexibility, owing to the abundance of Glycine and Serine. After insertion, the *BspEI* site is preserved on only one side although new intercalated sequences may be added successively. At each stage, the orientation is controlled by the *BspEI* enzyme. Two copies of this sequence are needed to restore the normal fluorescence of GFP, but the energy transfer between aequorin and GFP is optimal with five copies. The entire intercalated sequence of pGCA plasmid (5 x 9 aa + the five initial amino acids = 50 aa) was verified by sequencing:

	Lys	Ser	Gly	Leu	Arg	Ser	Val		(SEQ ID NO: 43)
5'	AAG	TCC	GGA	CTC	AGA	TCT	GTC	3'	(SEQ ID NO: 42)
3'	TTC	<u>AGG</u>	<u>CCT</u>	GAG	<u>TCT</u>	<u>AGA</u>	CAG	5'	(SEQ ID NO: 44)

GFP	<i>BspEI</i>		<i>BglII</i>	Aeq
-----	--------------	--	--------------	-----

↓

5'	AAG	T	GC	GGA	CTC	AGA	TCT	GTC	3' (SEQ ID NO: 45)
3'	TTC		AGG	CC	T	GAG	TCT	AGA	CAG 5' (SEQ ID NO: 44)
+									
	Gly	Gly	Ser	Gly	Ser	Gly	Gly	Gln	Ser (SEQ ID NO: 47)
5' CC	GGC	GGG	AGC	GGA	TCC	GGC	GGC	CAG	T 3' (SEQ ID NO: 46)
3' _____	G	CCC	TCG	<u>CCT</u>	<u>AGG</u>	CCG	CCG	GTC	<u>AGG</u> <u>CC</u> 5' (SEQ ID NO: 48)
			BamHI					BspEI	

# IN THE CLAIMS

Please amend the claims as follows:

1. (Amended) A method of screening *in vivo* for a change in a physical, chemical, biochemical, or biological, condition, the method comprising the steps of:
  - a) administering to a mammal [an acceptable] a composition [-] comprising a bioluminescent system [according to claims 1 to 6];
  - b) detecting whether light is produced; and
  - c) optionally measuring the ionic concentration of calcium flux.
2. (Amended) A composition comprising a purified peptide, wherein [said] **the** composition [has the functional characteristics to binding] **binds** calcium ions and [to permit] **transmits** [a measureable] **measurable** energy, [said] **wherein the amount of** energy [depending of] **depends on** the quantity of calcium bound and [of] the quantity of [polypeptides] **peptide** in [said] **the** composition in absence of any light excitation.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT,  
& DUNNER, L.L.P.  
1300 I STREET, N. W.  
WASHINGTON, DC 20005  
202-408-4000

3. (AMENDED) A purified polypeptide having the amino acid sequence of **any one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.**

9. (AMENDED) [An] A purified polynucleotide having the sequence of **any one of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, or SEQ ID NO: 12.**

15. (Amended) [A] **The** composition according to claim 2, wherein [said] **the** purified polypeptide [is a purified polypeptide according to any one of claims 3 to 18] **has the amino acid sequence of any one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.**

18. (Amended) A method of screening *in vitro* **for a molecule in a biological sample** capable of modulating the energy in [a] **the composition of claim 2** [according to claim 22], **wherein the molecule is contained in a reaction system**, wherein the method comprises:

[(a) providing in a biological sample a composition according to claim 22 in a reaction system comprising the molecule to be tested;]

[(b)] (a) detecting a modulation of the energy by comparison with a control sample containing [said] **the composition [according to] of claim [22] 2** without the molecule to be tested; and

(c) optionally, determining the effective minimal concentration of [said] **the molecule capable of inhibiting or increasing the energy transfer of [said] the composition.**

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT,  
& DUNNER, L.L.P.  
1300 I STREET, N. W.  
WASHINGTON, DC 20005  
202-408-4000

19. (AMENDED) A culture containing a polynucleotide [according to] **of claim [16] 9**, said culture as deposited at the C.N.C.M. and containing **any one of the plasmids [the plasmid] in deposit accession No. I-2507, I-2508, I-2509, I-2510, I-2511, I-2512, or I-2513.**

26 (AMENDED) A peptide linker [having the function after translation to approach] **that links** a donor site to an acceptor site [in optimal conditions] to permit a direct transfer of energy by chemiluminescence in a purified polypeptide [according to claims 3 to 8] **wherein the polypeptide has a sequence as claimed in claim 3.**

27. (AMENDED) A nucleotide linker having the nucleotide sequence of **any one of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17.**

32. (AMENDED) A polynucleotide linker [according to any one of claims 27 to 31], **comprising the sequence of any one of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17, wherein after translation, the polynucleotide linker links** [having the function after translation to approach] a donor site to an acceptor site [in optimal conditions] to permit a direct transfer of energy by Chemiluminescence Resonance Energy Transfer (CRET) in [a purified polypeptide according to] **the purified polypeptide of the composition** in claim 2.

33. (AMENDED) A [peptidic] **peptide** linker of at least 5 amino acids [and] comprising the amino acid sequence of **any one of SEQ ID No: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NO: 22.**

38. (AMENDED) A peptide linker [according to any one of claims 33 to 44, having the function] **comprising any one of SEQ ID No: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID**

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT,  
& DUNNER, L.L.P.  
1300 I STREET, N.W.  
WASHINGTON, DC 20005  
202-408-4000

**NO: 21, or SEQ ID NO: 22, wherein** after translation, **the linker links** [to approach] a donor site to an acceptor site [in optimal conditions] to permit a direct transfer of energy in the presence of [a] **the purified polypeptide of the composition** according to claim 2.

39. (AMENDED) A peptide linker according to [any one of claims] **claim 33** [to 37], **wherein the peptide linker** [which] has the capacity to stabilize a modified bioluminescent system *in vivo* and/or *in vitro*.

40. (AMENDED) A modified bioluminescent system comprising two bioluminescent proteins and a peptide linker according to [any one of claims] **claim 33** [to 39].

42. (AMENDED) [A] **The** modified bioluminescent system [according to claims 40 or 41] **of claim 40** comprising [by the following constituents:] aequorin protein and a GFP protein.

43. (AMENDED) A kit for measuring the transfer of energy *in vivo* or *in vitro* and containing at least one of the polypeptides [according to claims] **of claim 3** [to 8 or the polynucleotide according to claims 9 to 14] and the reagents necessary for visualizing or detecting the [said] transfer **of energy** in presence or in absence of a molecule of interest.

46. (AMENDED) The fusion protein as claimed in claim 45, wherein the LINKER comprises the following amino acids:

(Gly Gly Ser Gly Ser Gly Gly Gln Ser [[] (SEQ ID NO: [251] **25**) []])<sub>n</sub>,

wherein n is 1-5.

53. (AMENDED) A polynucleotide encoding a fusion protein as claimed in [any one of claims] **claim 44** [to 52].

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT,  
& DUNNER, L.L.P.  
1300 I STREET, N. W.  
WASHINGTON, DC 20005  
202-408-4000